

Cortactin Controls Cell Motility and Lamellipodial Dynamics by Regulating ECM Secretion

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Summary

Background: Branched actin assembly is critical for both cell motility and membrane trafficking. The branched actin regulator cortactin is generally considered to promote cell migration by controlling leading-edge lamellipodial dynamics. However, recent reports indicate that lamellipodia are not required for cell movement, suggesting an alternate mechanism.

Results: Because cortactin also regulates membrane trafficking and adhesion dynamics, we hypothesized that altered secretion of extracellular matrix (ECM) and/or integrin trafficking might underlie motility defects of cortactin-knockdown (KD) cells. Consistent with a primary defect in ECM secretion, both motility and lamellipodial defects of cortactin-KD cells were fully rescued by plating on increasing concentrations of exogenous ECM. Furthermore, cortactin-KD cell speed defects were rescued on cell-free autocrine ECM produced by control cells, but not on ECM produced by cortactin-KD cells. Investigation of the mechanism revealed that whereas endocytosed fibronectin (FN) is redeposited at the basal cell surface by control cells, cortactin-KD cells exhibit defective FN secretion and abnormal FN retention in a late endocytic/lysosomal compartment. Cortactin-KD motility and FN deposition defects were phenocopied by KD in control cells of the lysosomal fusion regulator synaptotagmin-7. Rescue of cortactin-KD cells by expression of cortactin-binding domain mutants revealed that interaction with the Arp2/3 complex and actin filaments is essential for rescue of both cell motility and autocrine ECM secretion phenotypes, whereas binding of SH3-domain partners is not required.

Conclusions: Efficient cell motility, promoted by cortactin regulation of branched actin networks, involves processing and resecretion of internalized ECM from a late endosomal/lysosomal compartment.

Introduction

Cell motility is a fundamental component of many physiological and pathological processes, including embryogenesis, wound healing, and cancer metastasis. Intrinsic cell motility cycles canonically consist of protrusion of leading-edge lamellipodia, formation of new adhesions, cell body contraction, and tail detachment. These cycles are modified by cellular interaction with extrinsic factors, including growth factors and extracellular matrix (ECM) [1]. A motility molecule that has received a great deal of attention is cortactin, as a result

of its presence in leading-edge lamellipodia and its function as a regulator of the actin-nucleating Arp2/3 complex [2]. Numerous studies have shown that cortactin regulates cell migration in diverse cell types [3], including *Drosophila* border cells migrating in vivo [4]. In contrast, some other reports have found little or no effect of cortactin expression on cell migration, suggesting either cell type or microenvironmental influences [5, 6].

The underlying mechanism for cortactin regulation of cell motility has been variably attributed to regulation of Arp2/3-mediated branched actin dynamics in leading-edge protrusions or to regulation of signaling [2, 7, 8]. However, a number of studies have found that cortactin is not essential for lamellipodial protrusion but rather affects lamellipodial dynamics [3]. In addition, lamellipodial protrusion has been shown to be dispensable for cell movement [9], suggesting that lamellipodial protrusion may serve primarily to direct the cell rather than drive the actual mechanics of intrinsic cell motility. By contrast, adhesion of cells to ECM is known to be critical for cell motility [1, 10].

Previously, we identified specific defects in both lamellipodial persistence (e.g., stability) and adhesion assembly in cortactin-knockdown (KD) cells [7] that were coordinately rescued along with cell motility by a minimal cortactin truncation protein that contained the Arp2/3 complex and F-actin binding sites. Interestingly, lamellipodial persistence defects are frequently associated with primary defects in integrin activity or expression [11–13], which suggests that the adhesion assembly defect of cortactin-KD cells might cause lamellipodial instability rather than vice versa. Because cortactin is known to regulate membrane trafficking [3], we hypothesized that the lamellipodial, adhesion, and motility defects observed in cortactin-deficient cells [3] could be a consequence of defective ECM secretion or altered integrin trafficking. Indeed, in this study we report that cortactin-KD cells exhibit decreased fibronectin (FN) secretion that leads to defects in cell motility and lamellipodial dynamics. Interestingly, investigation of the mechanism revealed that, dependent on cortactin interaction with branched actin networks, exogenous FN is internalized and resecreted from a late endocytic/lysosomal compartment to promote efficient cell motility.

Results

The goal of this study was to test the hypothesis that cortactin regulates cell motility by altering membrane trafficking of either ECM components or integrins. Such a mechanism would link two major cellular functions (migration and vesicular trafficking) described for cortactin and might explain divergent reports on the role of cortactin in cell motility.

Migration and Lamellipodial Defects of Cortactin-KD Cells Are Rescued on Exogenous ECM

If cortactin primarily regulates motility through autocrine secretion of ECM, then the defective motility of cortactin-KD cells should be rescued by performing cell motility assays on ECM-coated surfaces. To test this possibility, we coated serially diluted FN or collagen I on the bottom of tissue culture

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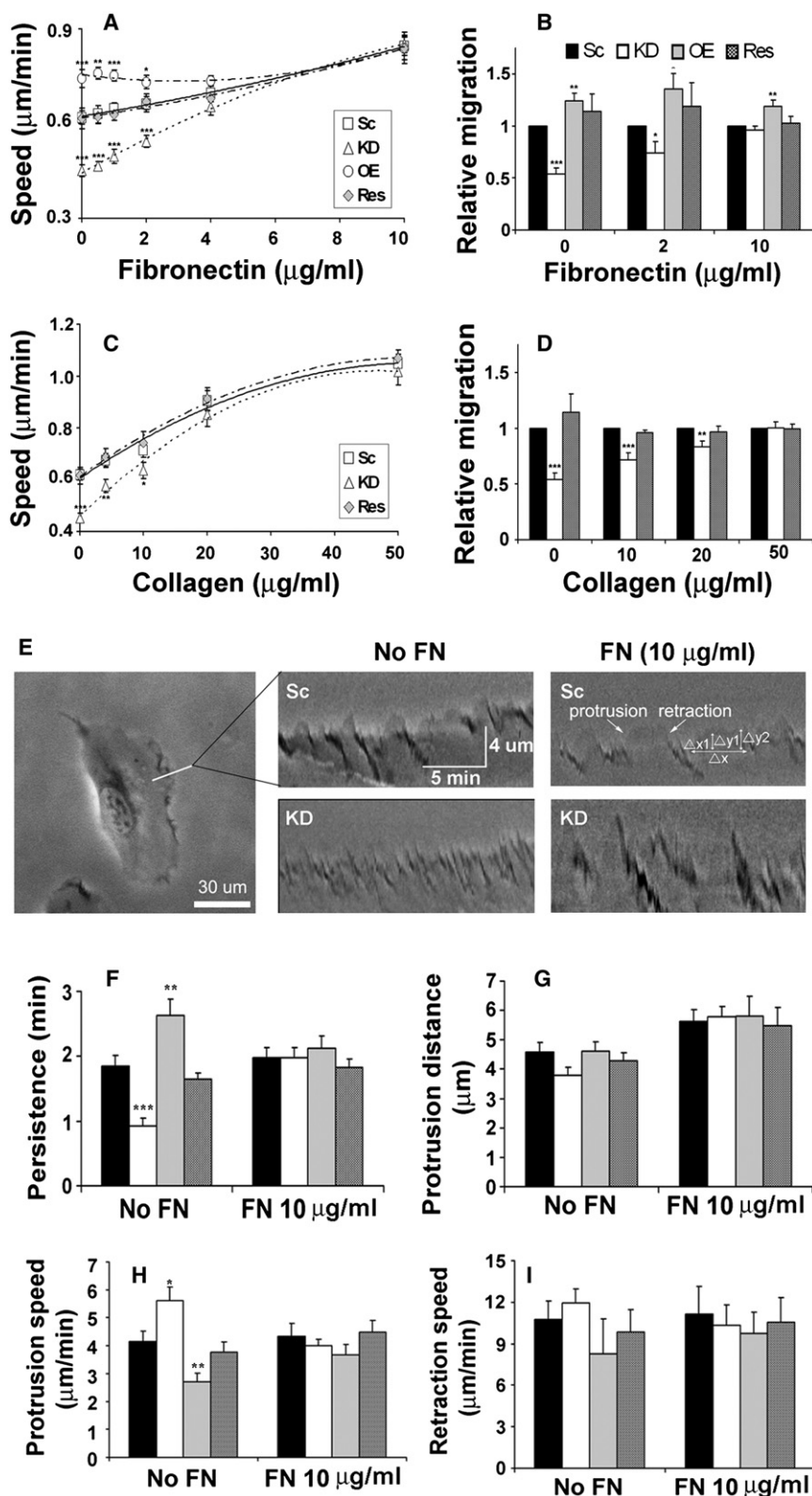


Figure 1. Cortactin-Knockdown Cell Motility and Lamellipodial Defects Are Rescued on Substrates Coated with Exogenous Extracellular Matrix

(A and C) Single-cell migration assays on fibronectin-coated (A) or collagen-coated (C) plates. The following abbreviations are used: Sc, scrambled oligo; KD, cortactin-knockdown; OE, cortactin-overexpressing; Res, rescued (KD cells re-expressing shRNA-insensitive mouse cortactin). $n \geq 45$ cells from three independent experiments for each cell line.

(B and D) Transwell migration assays on fibronectin-coated (B) or collagen-coated (D) transwell filters. Four different fields were observed in each of three independent experiments.

(E) Representative kymographs on uncoated or fibronectin-coated (FN; 10 $\mu\text{g}/\text{ml}$) plates. Note definitions of lamellipodial persistence (length of time that a protrusion lasts before retraction, Δx), protrusion distance (Δy_2), and protrusion speed (initial rate, $\Delta y_1/\Delta x$). Scale bars indicate distance and time axes.

(F–I) Quantitation of lamellipodial persistence (F), protrusion distance (G), protrusion speed (H), and retraction speed (I). $n \geq 20$ cells from three or more independent experiments.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to Sc. Error bars indicate standard error of the mean (SEM).

(scrambled) or KD cells rescued with short hairpin RNA (shRNA)-insensitive mouse cortactin cDNA (Res), whereas cortactin-overexpressing (OE) cells migrated faster (Figures 1A and 1B, 0 $\mu\text{g}/\text{ml}$ FN; see Figure S1A available online for cortactin protein abundance). However, plating cells on increasing concentrations of FN or collagen I led to full rescue of cortactin-KD motility defects to the level of control cells (Figures 1A–1D) at higher ECM concentrations. Similarly, single-cell motility defects of cortactin-KD MDA-MB-231 breast cancer cells were rescued by plating cells on the highest tested concentrations of FN or collagen I (Figure S1). There was much less effect of exogenous ECM on control and OE cell speed, suggesting a more optimal ECM-adhesion axis [10] on uncoated surfaces as might occur with autocrine secretion of ECM. These data suggest an extrinsic and not intrinsic motility defect in cortactin-KD cells.

We previously reported that cortactin-KD cells have defective stability and persistence of lamellipodial protrusions that parallel the motility defects [7]. To analyze the effect of exogenous ECM

plates for single-cell (Figures 1A and 1C) or transwell membranes for transwell (Figures 1B and 1D) migration assays. As reported previously [7], in the absence of exogenous ECM, cortactin-KD HT1080 fibrosarcoma cells migrated less efficiently than scrambled oligo-expressing control cells

on lamellipodial dynamics, we recorded membrane ruffling of cortactin-manipulated HT1080 cells plated on 0 or 10 $\mu\text{g}/\text{ml}$ FN in time-lapse movies and performed analysis by kymography [7, 14] (Figure 1E). Consistent with the migration assays, the lamellipodial persistence defect of cortactin-KD cells [7]

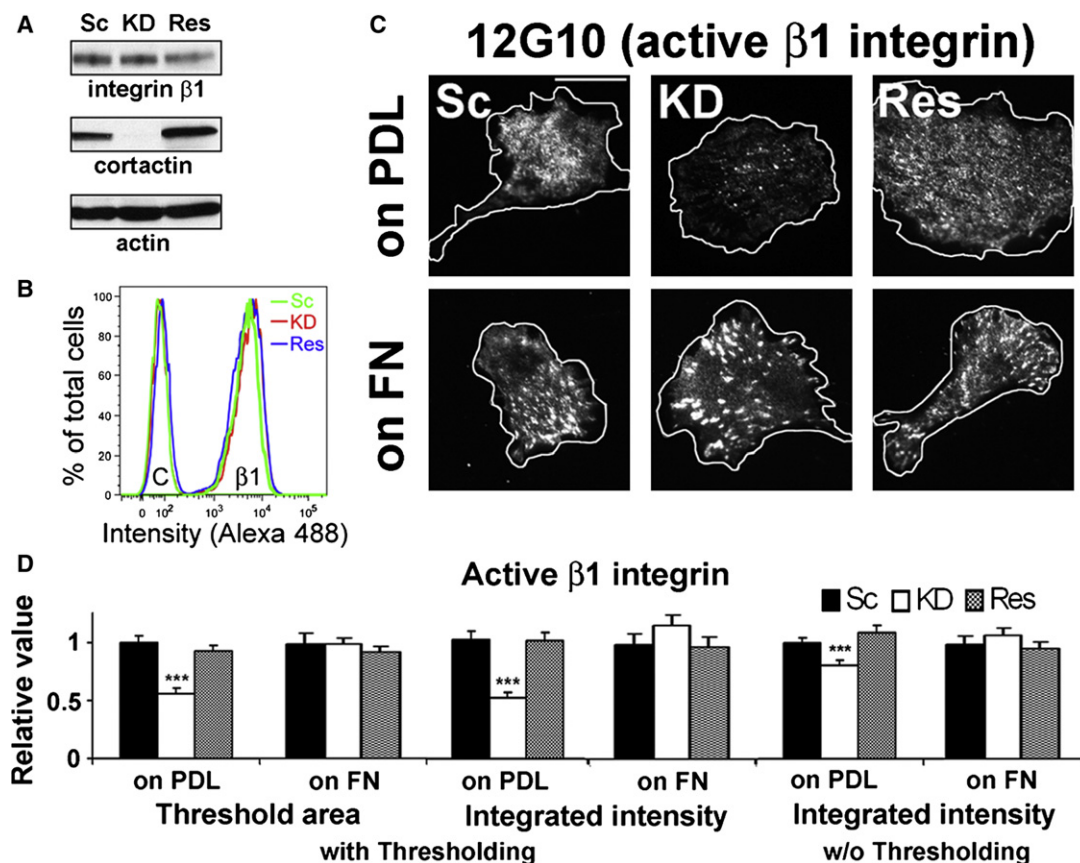


Figure 2. Cortactin Expression Affects Adhesion Formation on Low-Extracellular Matrix Substrates but Not Total or Cell Surface Levels of $\beta 1$ Integrin
(A) Western blot analysis of total cell lysates from scrambled control (Sc), cortactin-KD (KD), and cortactin-rescued (Res) HT1080 cells shows no difference in total cell $\beta 1$ integrin levels. Representative blot from $n = 2$ experiments.
(B) Flow cytometry analysis shows no difference in $\beta 1$ cell-surface expression. Representative profiles are shown. The following abbreviations are used: C, control IgG; $\beta 1$, integrin $\beta 1$. $n = 2$ experiments.
(C) Representative TIRF images of activated (visualized using 12G10 mAb staining) integrin $\beta 1$ at basal surface of cells cultured on poly-D-lysine-coated (PDL; 100 $\mu\text{g}/\text{ml}$) or FN-coated (10 $\mu\text{g}/\text{ml}$) glass surfaces. Cell boundaries from differential interference contrast (DIC) images (Figure S2) are outlined in white. Scale bar represents 30 μm .
(D) Quantitation of activated integrin $\beta 1$ -positive area per cell and intensity per cell calculated with or without (w/o) thresholding of TIRF images. $n > 30$ cells from three independent experiments. *** $p < 0.001$. Error bars indicate SEM.

was fully rescued on 10 $\mu\text{g}/\text{ml}$ FN (Figure 1F). Exogenous FN also normalized the increased protrusion speed of cortactin-KD cells [7] to the level of controls (Figure 1H). Protrusion distance and retraction speed were not significantly different between cortactin-manipulated cells in either the absence [7] or presence of exogenous FN (Figures 1G and 1I). Alterations in lamellipodial dynamics with OE cells were also equalized to the level of scrambled controls by plating on 10 $\mu\text{g}/\text{ml}$ FN.

HT1080 cells are known to express integrin receptors $\alpha 2\beta 1$ for collagen I, $\alpha 3\beta 1$ for laminin, and $\alpha 5\beta 1$ for FN [15]. Because cortactin-regulated motility was sensitive to the levels of the $\beta 1$ integrin ligands collagen I and FN, we tested whether $\beta 1$ expression was altered. The total cellular level of $\beta 1$ integrin was similar between control and cortactin-KD cells by western blot analyses (Figure 2A). In addition, flow cytometry revealed no differences in cell-surface expression of $\beta 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$ integrins (Figure 2B; Figure S2A). We also quantitated the presence of $\beta 1$ integrin in adhesions in cortactin-manipulated cells plated on the non-integrin-binding attachment substrate poly-D-lysine (PDL) or 10 $\mu\text{g}/\text{ml}$ FN by immunostaining fixed, permeabilized cells for the presence of total (AlIB2) or activated (12G10) $\beta 1$ and imaging the cell-substrate interface with total

internal reflection fluorescence (TIRF) microscopy. Consistent with the motility and lamellipodial dynamics results, cortactin-KD cells had reduced total and activated $\beta 1$ present in adhesions when plated on PDL, but not on FN (Figure 2; Figure S2). Analysis of $\beta 1$ integrin endocytosis exhibited a similar profile, with an increase in early $\beta 1$ internalization in cortactin-KD cells when plated on low but not high FN (2 and 5 min time points, Figure S2). We speculate that the increased endocytosis of $\beta 1$ integrin in cortactin-KD cells plated on low concentrations of FN is likely due to defective adhesion formation (Figure 2; Figure S2; [7]), leading to a larger population of unengaged $\beta 1$ integrins available for endocytosis [16]. Because both the low adhesion formation and the increase in early $\beta 1$ endocytosis were rescued when cortactin-KD cells were plated on high FN concentrations, we hypothesize that the primary underlying defect in cortactin-KD cells is defective secretion of ECM.

Motility Defects of Cortactin-KD Cells Are Due to Defective Secretion of Autocrine ECM

To directly test the effect of autocrine-produced ECM on cell motility, we prepared tissue culture plates coated with cell-free autocrine ECM from cortactin-manipulated HT1080

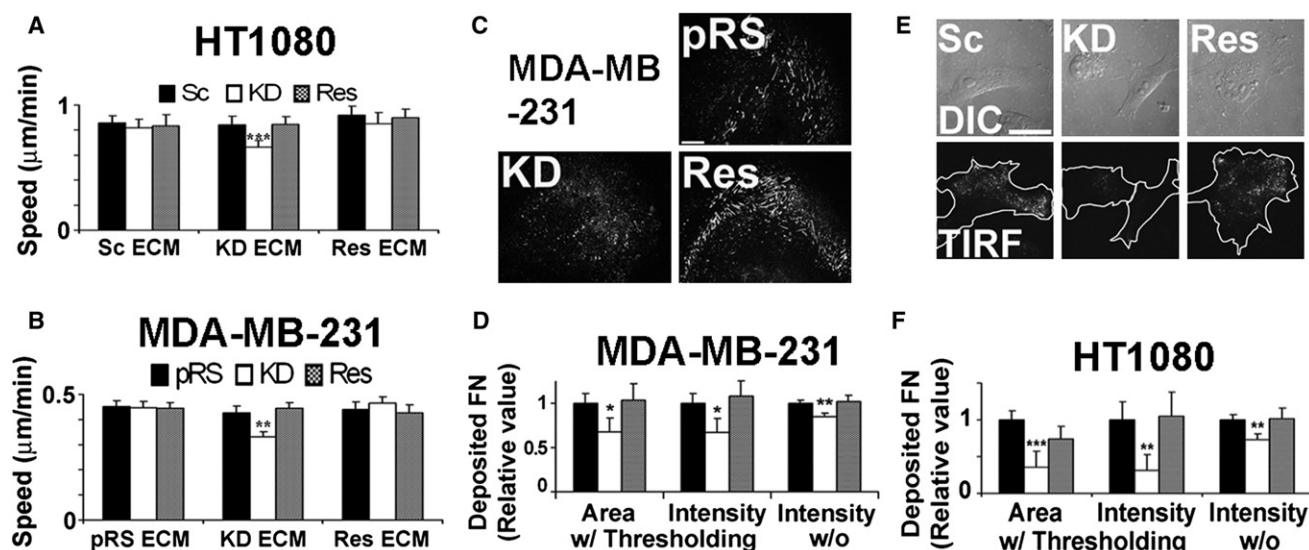


Figure 3. Autocrine-Produced Extracellular Matrix from Cortactin-Expressing Cells Rescues the Motility Defect of Cortactin-KD Cells
(A and B) Single-cell migration assays of cortactin-manipulated HT1080 (A) and MDA-MB-231 (B) cells plated on cell-free autocrine extracellular matrix (ECM) derived from control or KD cells as indicated. The following abbreviations are used: Sc, scrambled control; pRS, vector-expressing; KD, cortactin-KD; Res, cortactin-rescued. $n > 50$ cells from three independent experiments.
(C) Representative images of FN immunostaining of cell-free ECM derived from cortactin-manipulated MDA-MB-231 cells. Scale bar represents 30 μ m.
(D) Quantitation of the area per frame and intensity per frame of MDA-MB-231-derived cell-free ECM images. $n > 30$ images from three independent experiments.
(E and F) Analysis of basally secreted FN in HT1080 cells.
(E) Representative matched DIC and TIRF images from fixed, immunostained cortactin-manipulated HT1080 cells showing a deficit in basal FN underneath cortactin-KD cells. Scale bar represents 30 μ m.
(F) Quantitation of FN-positive area per cell and intensity per cell from TIRF images. $n \geq 25$ images from three independent experiments.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to control cells. Error bars indicate SEM.

cells. Confluent cultures were grown for 48 hr on 12-well plates before removal of cells with 20 mM ammonium hydroxide (NH_4OH). NH_4OH is minimally damaging to autocrine-produced ECM and leaves many biological properties intact, including adhesion, spreading, and growth [17]. New cultures of cortactin-manipulated HT1080s were then plated on the autocrine-ECM-coated dishes for 2 hr before testing in 5 hr single-cell motility assays. As predicted, cortactin-KD cell motility was fully rescued on autocrine ECM extracted from scrambled and rescue cells, but not on cortactin-KD-derived autocrine ECM (Figure 3A). However, the motility of scrambled and rescue cells was similar regardless of the source of autocrine ECM, suggesting that cortactin-expressing cells can sufficiently modulate the ECM environment during the experiment for efficient migration. Cortactin-manipulated MDA-MB-231 cells responded similarly, with rescue of KD motility on autocrine-produced ECM from cells expressing cortactin (Figure 3B).

Immunofluorescent staining for FN in the autocrine-produced cell-free ECM revealed little detectable deposition in HT1080-produced ECM; however, FN staining was detectable in MDA-MB-231-produced ECM, and there was less FN deposition in cortactin-KD cell-produced ECM (Figures 3C and 3D). To verify this finding in a second cell type, we obtained previously published control and cortactin-KD mouse embryonic fibroblasts (MEFs) [18] and prepared cell-free ECM. As expected, there was less FN detected in autocrine ECM prepared from cortactin-KD MEFs, compared with control-derived ECM (Figure S3). We speculate that the inability to visualize HT1080-produced cell-free FN represents either a signal-to-noise issue or greater extractability of

HT1080 ECM from glass coverslips by NH_4OH , because we visualized it easily below cortactin-manipulated HT1080 cells by TIRF microscopy (Figures 3E and 3F). Note also that the cell motility experiments were performed on autocrine-produced ECM-coated plastic, and plastic adsorbs ECM more efficiently than glass [19]. These data indicate that cortactin is critical for FN secretion at the cell-substrate interface.

Cortactin Regulates Secretion of FN from an Endocytic Compartment

To determine whether changes in FN expression could explain the decreased FN secretion into cortactin-KD-produced autocrine ECM, we performed quantitative RT-PCR and western blot analyses. No significant differences were observed in total cell levels of FN mRNA or protein between any of the cortactin-manipulated cell lines (Figures 4A and 4B), suggesting that cortactin may instead regulate FN trafficking. Indeed, examination of permeabilized cells immunostained for FN revealed that twice as many cortactin-KD cells contained large perinuclear FN-containing puncta as compared with control cells (Figure 4C, top panels), suggesting an exit defect from a secretory compartment.

To identify that compartment, we first tested whether the major source of secreted FN was exogenous (i.e., the serum contains significant amounts of FN [20]) as opposed to biosynthetic pathways. Up to this point, all experiments had been performed in media containing regular serum. To determine the predominant pathway in our system, we repeated two critical experiments in media containing FN-depleted serum: immunostaining of intracellular FN and single-cell motility on autocrine-produced ECM. For immunostaining, cells were

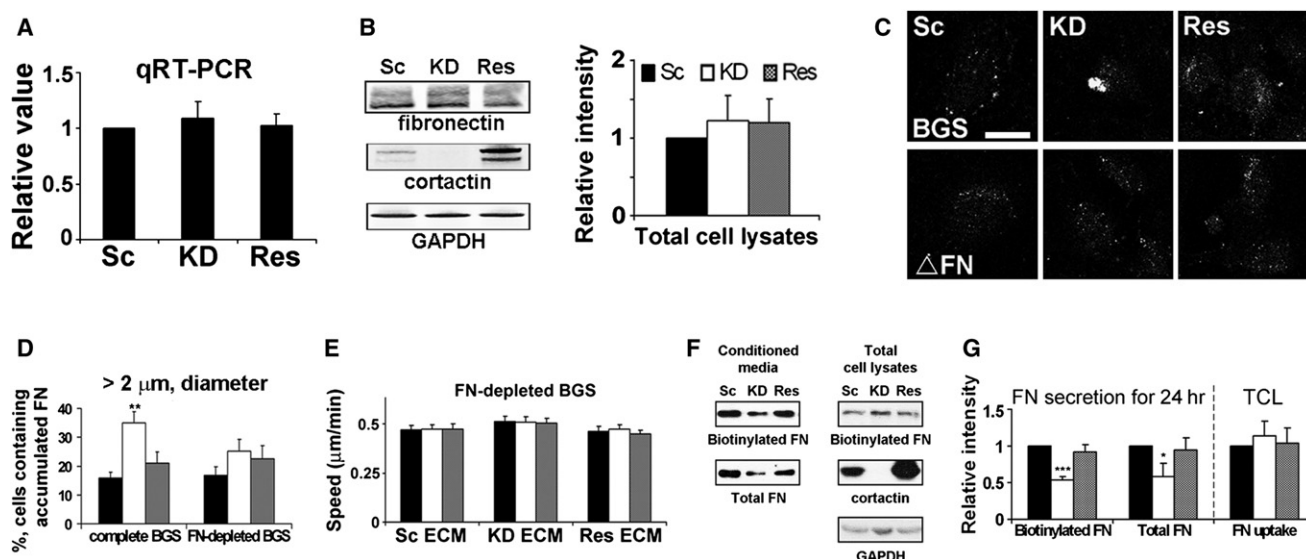


Figure 4. Internalized Fibronectin Is Used for Motility

(A) Quantitative real-time PCR. Relative FN mRNA abundance was measured by $2^{-\Delta\Delta C_t}$. $n = 3$ experiments.
(B) Representative western blot images and analyses of FN protein abundance in total cell lysates. FN intensities were normalized by GAPDH intensities. $n = 3$ experiments.
(C and D) To analyze intracellular accumulation of ECM, cortactin-manipulated HT1080 cells were cultured in media containing complete bovine growth serum (BGS) or FN-depleted BGS (Δ FN) and immunostained for FN.
(C) Representative fluorescence confocal images. Scale bar represents 30 μ m.
(D) Percentage of cells with large intracellular FN-positive puncta (≥ 2 μ m diameter). $n \geq 200$ cells from three independent experiments.
(E) Single-cell migration on cell-free autocrine ECM with FN-depleted BGS-containing media (compare to Figure 3A). $n \geq 50$ cells from three independent experiments.
(F) Representative western blot images from pulse-chase assay performed using poly-HRP-conjugated streptavidin ("biotinylated") and FN antibody ("total"). Total cell lysates were gathered after the 1 hr pulse to show biotinylated FN uptake. Conditioned media were gathered 24 hr after the pulse chase.
(G) Quantitation of experiments in (F). TCL indicates total cell lysates and represents biotinylated FN signal normalized by the GAPDH loading control. $n = 3$ experiments.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to control cells. Error bars indicate SEM.

plated on PDL-coated coverslips for 48 hr in media containing 10% bovine growth serum (BGS) or 10% FN-depleted BGS (Figure S4) followed by fixation (Figures 4C and 4D). For motility experiments, cell-free autocrine ECM was prepared as before, cells were plated in media containing FN-depleted BGS and equilibrated for 2 hr, and movies were then taken for 5 hr (Figure 4E). Interestingly, intracellular FN accumulation in cortactin-KD cells was decreased and equivalent to control cells upon culturing in FN-depleted BGS (Figures 4C and 4D). Likewise, single-cell motility of all cortactin-manipulated HT1080 cells was equivalent and dampened (by almost 2-fold) when the experiment was performed in FN-depleted BGS (Figure 4E; compare to Figure 3A). These data indicate that, dependent on cortactin, cells internalize and resecret exogenous ECM to facilitate effective migration.

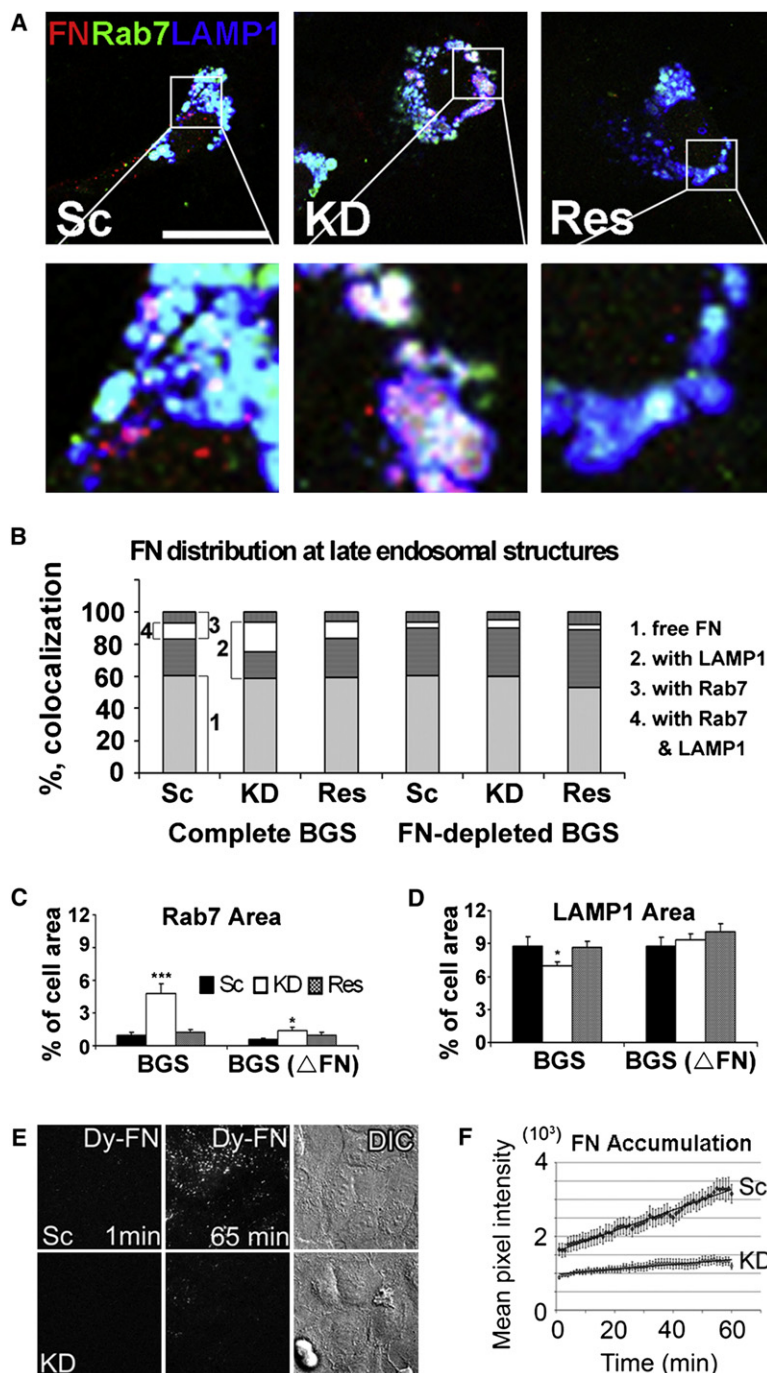
Because our motility experiments indicated that internalized FN is critical for cortactin-regulated motility, we performed a pulse-chase experiment to determine whether cortactin affects resecretion of internalized FN into conditioned media. HT1080 cells were incubated with 20 μ g/ml biotinylated FN for 1 hr followed by washing and collection of conditioned medium after 24 hr. Indeed, despite equivalent FN uptake, cortactin-KD cells secreted 2-fold less biotinylated FN into conditioned medium compared with controls (Figures 4F and 4G).

Cortactin Regulates a Late Endosomal/Lysosomal Secretory Compartment

To determine which secretory compartment is regulated by cortactin, we colocalized internalized FN with vesicular

markers associated with recycling endosomes, apicobasal trafficking, and secretory lysosomes [21]. Whereas there was little colocalization of FN with Rab8a or Rab11a (Figure S5A), a substantial proportion of the internalized FN colocalized with the late endosomal marker Rab7 and the lysosomal marker LAMP1 (Figures 5A and 5B). Interestingly, cortactin-KD cells exhibited a significant increase in colocalization of FN with Rab7-positive and Rab7/LAMP1 double-positive compartments compared with control cells (Figures 5A and 5B; Figures S5B and S5C). There was also a large increase in the size of the Rab7 compartment in cortactin-KD cells (Figure 5C), suggesting a block in secretion from and/or maturation of late endosomes. There was also a small decrease in the size of the LAMP1-compartment (Figure 5D), suggesting a shift in the overall late endosomal/lysosomal population upon cortactin manipulation. Cortactin was also found to colocalize with Rab7- and LAMP1-positive vesicles (Figure S5D). Most KD phenotypes, including FN accumulation in Rab7-positive compartments, were rescued or diminished by removal of FN from the media (FN-depleted BGS condition, Figures 5B–5D), consistent with an exogenous source of accumulated FN.

To provide additional evidence that FN derived from the media and processed by the endocytic system is deposited at the basal surface of cells, we incubated cells with DyLight 550-conjugated FN and performed live-cell imaging. By confocal microscopy, DyLight-FN localized to acidic organelles labeled with LysoTracker Green (Figure S5E). By TIRF microscopy, DyLight-FN was visible in moving vesicles and



also appeared to be deposited at the basal surface of cells, as evidenced by progressive accumulation in elongated immobile structures that may represent newly assembling adhesions (Movie S1; Movie S2). In addition, a few of the elongated structures disappeared before the end of recording, indicating either that nascent FN deposits were re-endocytosed or that they represented tethered lysosomes that had not yet fused with the plasma membrane (Movie S1; Movie S2). Compared with control cells, there was an ~4-fold slower rate of total Dy-Light-FN accumulation at the base of cortactin-KD cells (Figures 5E and 5F). Size and shape analyses of the movie data indicated that KD cells exhibited a significant reduction

Figure 5. Cortactin Promotes Fibronectin Trafficking from a Late Endosomal/Lysosomal Secretory Compartment

(A–D) Colocalization of FN with vesicular compartments. (A) Representative confocal images of FN (red), Rab7 (late endosomes, green), and LAMP1 (lysosomes, blue) triple staining of cells cultured in complete BGS-containing media. White indicates triple colocalization; magnifications of boxed areas are shown below. Scale bar represents 30 μ m. (B) Summary graph of FN localization shows accumulation of FN in a Rab7/LAMP1 double-positive compartment in KD cells. (C) Area of Rab7 staining out of total cell area (from single confocal slices). (D) Area of LAMP1 confocal staining out of total cell area. Error bars in (C) and (D) indicate SEM from three independent experiments ($n \geq 50$ cells total). * $p < 0.05$; *** $p < 0.001$ compared to control cells. (E and F) Cortactin is necessary for accumulation of endocytosed DyLight 550-FN at the cell-substrate interface. (E) Frames from TIRF live-cell imaging sequences taken at one frame per minute (see also Movie S1 [control] and Movie [KD]). The first frame of the movie (time 0) has been subtracted from each frame of the time sequence in order to detect only newly accumulated FN. Note efficient accumulation for Sc as compared to KD cells. DIC images illustrate confluence of cell monolayers. (F) Quantitation of live-cell imaging data as in (E). FN accumulation was quantitated as mean fluorescence intensity beneath ten individual cells from four independent experiments and was significantly different between Sc and KD cells ($p < 0.001$, assessed by MatLab analysis of covariance comparing the two regression lines). Both Sc and KD cells exhibit constant FN accumulation (line function fitting confirmed by $R^2 > 0.9$). Slope coefficient is 27.965 for Sc and 6.56 for KD. Error bars indicate SEM.

in the number of all types of FN structures appearing in the TIRF plane, including both large linear accumulations and small circular objects (Figures S5F and S5G; Movie S1; Movie S2). These data, in combination with our finding that in cortactin-KD cells FN accumulates in an enlarged Rab7-positive compartment (Figures 5A–5C), suggest that cortactin is important for trafficking of endocytosed FN to the plasma membrane.

To test the general mechanism of lysosomal secretion in ECM deposition and cell motility, shRNA targeting the secretory lysosome fusion regulator synaptotagmin-7 (Syt7) was expressed in HT1080 cells. Indeed, similar to cortactin-KD, Syt7-KD HT1080 cells exhibited decreased basal FN deposition and decreased motility on uncoated tissue culture dishes compared with control cells (Figure 6). Also similar to cortactin-

KD cells, the motility of control and Syt7-KD cells was equivalent and increased on 10 μ g/ml FN-coated dishes.

Interaction of Cortactin with the Arp2/3 Complex and Actin Filaments Is Critical for Motility and Secretion Phenotypes

To determine underlying molecular interactions that mediate cortactin regulation of FN secretion and cell motility, we performed rescue experiments in which cortactin proteins with mutations in the binding sites for the Arp2/3 complex (W22A), actin filaments (Δ RP), or SH3-domain binding partners (W525K) or in Src (3Y) or Erk (S405A and S418A) phosphorylation sites [7, 22–24] were re-expressed in KD cells.

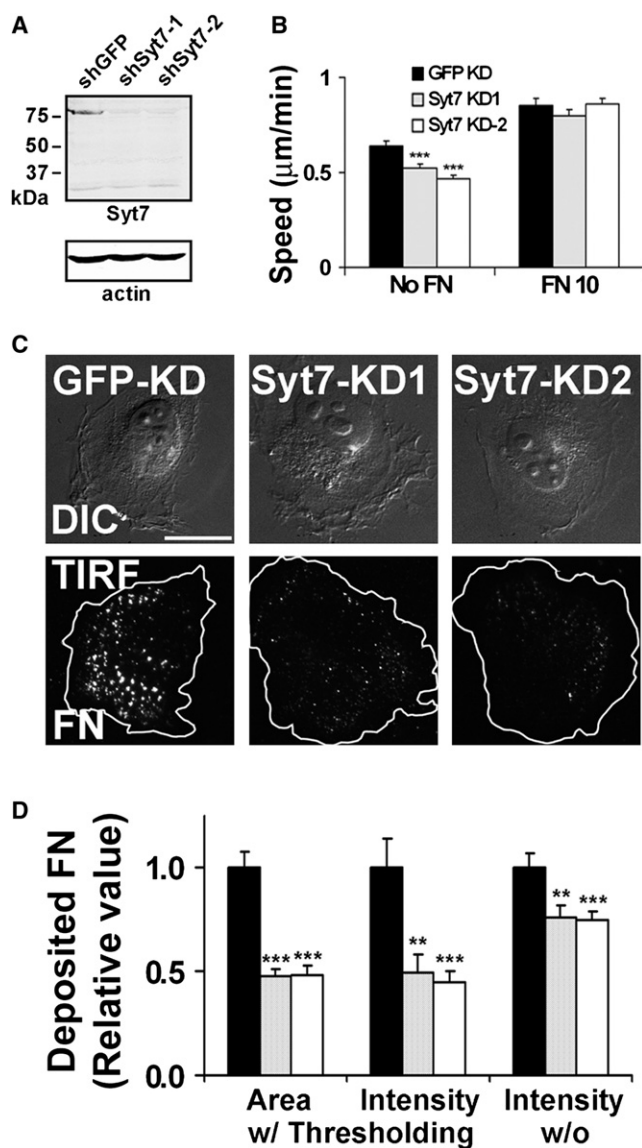


Figure 6. Loss of the Lysosomal Secretion Regulator Synaptotagmin-7 also Results in Defective Basal Fibronectin Secretion and Motility

(A) Western blot of synaptotagmin-7 (Syt7) expression in control (shGFP) and Syt7-KD (shSyt7-1 and shSyt7-2) HT1080 cells.

(B) Single-cell migration assays on uncoated (no FN) or FN-coated (FN 10; 10 $\mu\text{g}/\text{ml}$) plates. $n \geq 45$ cells for each cell line from three independent experiments.

(C) Representative matched DIC and TIRF images from fixed, immunostained Syt7-manipulated HT1080 cells showing a deficit of basal FN underneath Syt7-KD cells. Cell boundaries are outlined in white in TIRF images. Scale bar represents 30 μm .

(D) Quantitation of basal FN-positive area per cell or intensity per cell with (w/) or without (w/o) thresholding. $n > 30$ cells from three independent experiments.

*** $p < 0.01$; **** $p < 0.001$ compared to shGFP control. Error bars indicate SEM.

Interestingly, both cell motility and FN deposition defects of KD cells were rescued by re-expression of W525K and 3Y mutants (Figure 7; Figure S6). The rescue by W525K was frequently not to the level of that by wild-type cortactin or 3Y, although the differences were not statistically significant. Conversely, KD defects were not rescued by W22A, ΔLRP ,

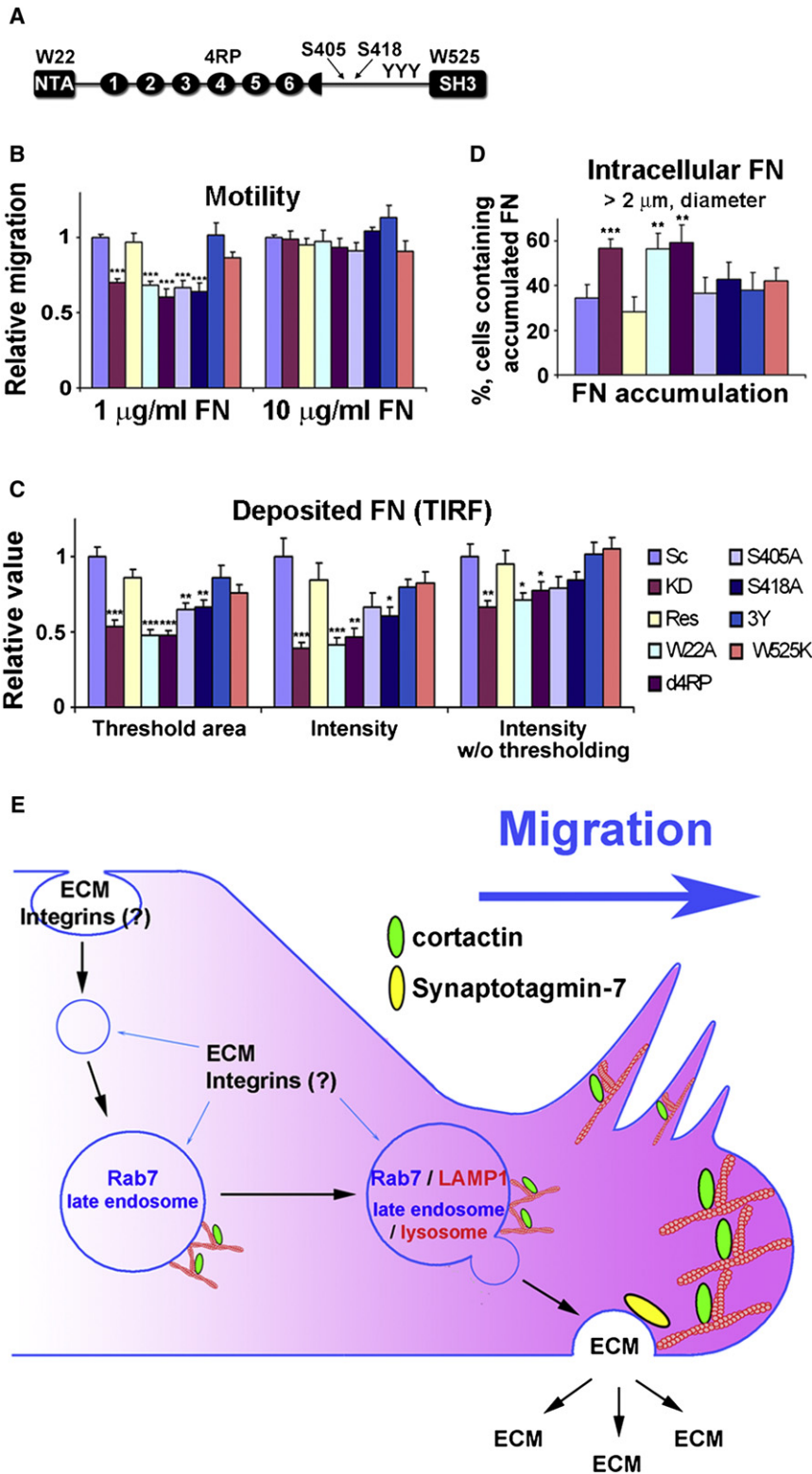
S405A, or S418A in either assay. Analysis of intracellular accumulation of large FN puncta yielded similar results, except that the Erk mutants also rescued KD defects in this assay (Figure 7; Figure S6). Our interpretation of these results is that the most critical activity of cortactin in FN secretion and cell motility is to regulate branched actin networks. A secondary (nonessential) activity may be to recruit or otherwise interact with SH3 binding partners at those networks.

Discussion

Cortactin regulates cell migration; however, diverse underlying mechanisms have been proposed. In this study, we report the novel finding that cortactin regulates extrinsic rather than intrinsic mechanisms of cell motility by promoting ECM secretion from a late endosomal/lysosomal compartment (see model in Figure 7E). Consistent with lysosomal secretion of ECM as a critical regulatory point for cell motility, cortactin-KD motility and secretion defects were phenocopied by KD of the late endosomal/lysosomal fusion regulator Syt7. Cortactin functions in motility and secretion were dependent on interaction with branched actin networks but not the SH3 domain.

Cortactin has been intensively studied as a motility factor since the discovery that it can serve as a cofactor for Arp2/3 activation as well as a stabilizer of actin branches [25, 26]. Numerous studies have demonstrated that cortactin promotes cell motility as well as proteolytic invasion through ECM [3]. However, conflicting results between studies suggest context-dependent cortactin functions. For example, several studies have reported no effect of cortactin loss on cell migration [6] or lamellipodial architecture [8]. Interestingly, the cell migration and electron microscopy studies in those reports were respectively performed using cells plated on 10 $\mu\text{g}/\text{ml}$ [6] and 50 $\mu\text{g}/\text{ml}$ [8] FN. Consistent with those results, we find that cortactin-KD cell defects in motility and lamellipodial dynamics are fully rescued to the level of control cells upon plating on similar concentrations of ECM. Our discovery that cortactin regulates cell motility by promoting ECM deposition provides a unifying mechanism that explains disparate literature findings and links reported cortactin functions in motility and membrane trafficking.

Through mutant rescue studies, we tested the requirements for key molecular interactions in cortactin regulation of FN trafficking and cell motility. Consistent with our previous report [7], we find that cortactin binding to the Arp2/3 complex and actin filaments is essential for rescue of all phenotypes. The other sites examined were in the C terminus and regulate binding of additional partners. Neither the SH3 domain, which binds most known cortactin binding partners, or the Src/Abl phosphosites, which create an SH2 binding domain when phosphorylated [3] and are thought to negatively regulate the SH3 domain [23], were required for rescue of any cortactin-KD phenotype. These data were somewhat surprising because several SH3-domain binding partners are good candidates for regulating vesicle trafficking, including the pin-chase dynamin 2, the cdc42 GEF Fgd1, and the Arf GAP ASAP1 [3]. By contrast, intact Erk phosphorylation sites are required for rescue of motility [27] and FN deposition, suggesting that Erk phosphorylation may regulate more cortactin functions than just SH3-domain accessibility [23, 28]. Based on the requirement for intact Arp2/3- and F-actin binding domains but not SH3-domain binding partners, we speculate that the truly essential role of cortactin in ECM trafficking is to regulate branched actin networks at late endosomes or at docking sites



for secretory endosomal/lysosomal carriers. Binding of SH3-domain partners by cortactin might contribute to cortactin-regulated vesicular trafficking events (e.g., fission or fusion) but may not be essential if a stable actin network in conjunction with membrane-bound signaling factors is sufficient to assemble the required multiprotein trafficking complexes.

examined as a cargo, and there are multiple clathrin-independent pathways [37]. Furthermore, cortactin likely does not affect all endocytic events, because dextran uptake is apparently unperturbed by cortactin inhibition [30].

A key finding of this study is that FN that is internalized, processed, and resecreted via a late endocytic/lysosomal

A somewhat surprising finding from this study is that cortactin-KD does not inhibit endocytosis of $\beta 1$ integrin or, apparently, FN. A number of studies have examined the role of cortactin in endocytosis, and most have found that KD or inhibition of cortactin reduces endocytic rates [29–36]. However, the vast majority of those studies examined clathrin-dependent endocytosis, and $\beta 1$ integrin has been reported to undergo clathrin-independent, lipid-raft-dependent endocytosis [16, 37]. Although cortactin has also been reported to regulate clathrin-independent endocytosis [32, 34], $\beta 1$ integrin has not been previously

compartment is reused as a motility substrate. Lysosomally processed FN may be particularly potent as a substrate, because partial degradation of FN can yield fragments with increased adhesiveness [38], and the lysosomal proteinase cathepsin produces biologically active fragments of FN [39]. To directly test this potential mechanism, we knocked down the lysosomal fusion regulator Syt7. Indeed, KD of Syt7 phenocopied cortactin-KD and inhibited both basal FN secretion and cell motility. Interestingly, a recent report implicated Syt7-mediated lysosomal secretion in leukocyte chemotaxis, and another study described regulation of epithelial cell migration by the late endosomal/lysosomal v-SNARE VAMP7 [40, 41], suggesting that this mechanism of motility regulation may be general. Both studies showed localization of lysosomal markers at lamellipodia, consistent with exocytosis occurring at the leading edge of migrating cells.

Although we focused on fibronectin secretion in this study, it is likely that the same vesicular carriers contain other motility-regulating molecules (Figure 7E). Indeed, integrins may be cosecreted with their ligands, because $\alpha 5 \beta 1$ integrin-FN cotrafficking through multivesicular bodies to lysosomes was recently shown to be important for cell migration [42]. Consistent with that idea, in our $\beta 1$ integrin endocytosis assays, the only change in KD cells that was not rescued by plating on high FN was intracellular accumulation of $\beta 1$ integrin at the late 15 min time point, suggesting a block in late endosomal trafficking. It is also possible that newly synthesized motility molecules may be secreted via the same pathway, because post-Golgi packaging can occur in endosomes and secretory lysosomes [43, 44].

In light of our findings, how do we consider the role of cortactin throughout the cell? Cortactin is one of the best markers of branched actin at various cellular sites and is particularly popular as a marker for branched-actin-rich lamellipodia and invadopodia [2]. Given the affinity of cortactin for the Arp2/3 complex [25] and newly polymerized actin [7] and the cooperative regulation of branched actin assembly noted by many investigators [26, 45–48], it seems likely that cortactin does regulate branched actin in lamellipodia and elsewhere. In fact, cortactin has been shown to affect cell edge protrusion during initial cell spreading on ECM-coated substrates [18], suggesting that cortactin does indeed regulate branched actin dynamics within cellular protrusions [7, 8, 49]. However, we found that interaction of cortactin with the Arp2/3 complex and F-actin is also required for FN secretion, suggesting that regulation of exocytosis is a key consequence of cortactin action on branched actin networks that promotes migration and invasion [3].

Experimental Procedures

Cell Culture and Gene Manipulation

HT1080 cell lines, knockdown and rescue constructs, and motility and lamellipodial dynamics assays were as described previously [7]. Cortactin mutants were gifts from Thomas Parson (University of Virginia) and were cloned into LZRS retroviral vector. A lentiviral shRNA expression system, pLKO.1, was used to knock down synaptotagmin-7 (Open Biosystems RHS3979-9577052 for KD-1 and RHS3979-9577053 for KD-2) or GFP (Sigma-Aldrich SHC005) in HT1080 cells.

Antibodies and Reagents

Anti- $\beta 1$ (12G10 for flow cytometry and M-106 for western blots), anti- $\alpha 2$ (P1E6), anti- $\alpha 3$ (P1B5), and anti- $\alpha 5$ (P1D6) integrin antibodies and normal mouse IgG₁ isotype control for fluorescence-activated cell sorting (sc-3877), anti-FN rabbit polyclonal (H-300 used in the triple staining with

Rab7 and LAMP1 antibodies), and anti-Rab7 (goat, A-16) antibodies were from Santa Cruz Biotechnology. Anti-cortactin mouse monoclonal (4F11, Upstate) and rabbit polyclonal (H-191, Santa Cruz Biotechnology), anti- β -actin (Ac-74, Sigma), anti-GAPDH (14C10, Cell Signaling), anti-FN mouse monoclonal (610077, used in TIRF and intracellular accumulation assays), anti-EEA1 (610456), and anti-CD107a (LAMP1, mouse, 555798) (BD Transduction Laboratories) antibodies were purchased from the listed manufacturers. Anti- $\beta 1$ integrin rat monoclonal antibody (A1B2) was a gift from Roy Zent (Vanderbilt University). Anti-Rab8a and anti-Rab11a antibodies were gifts from James Goldenring (Vanderbilt University). Anti-synaptotagmin 7 antibody (105 173) was from Synaptic Systems. Alexa Fluor-conjugated secondary antibodies were from Invitrogen. Human plasma FN was from GIBCO BRL, and human collagen type I was from Sigma. DyLight 550 NHS ester (62262) and EZ-Link sulfo-NHS-LC-LC-biotin (21338) were from Thermo Scientific and were conjugated to FN following the manufacturer's instructions. LysoTracker Green DND-26 (L-7526) was from Molecular Probes.

Statistical Analyses

All statistical analyses were performed using Student's *t* test and calculated by Microsoft Excel software, except for the live TIRF imaging, for which statistical analysis was performed using MatLab.

For more materials and methods, see [Supplemental Experimental Procedures](#).

Supplemental Information

Supplemental Information includes six figures, Supplemental Experimental Procedures, and two movies and can be found with this article online at [doi:10.1016/j.cub.2011.06.065](https://doi.org/10.1016/j.cub.2011.06.065).

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